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棉毛橐吾和穗序橐吾的自然杂交研究*

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摘要: 在棉毛橐吾 (*Ligularia vellerea*) 和穗序橐吾 (*L. subspicata*) 同域分布的居群中存在形态特征介于两者之间的个体。经分析, 这些个体有 3 种不同的形态类型, 可能是这两个物种之间的杂交后代。为检验这一假设, 对所采集的假设亲本和中间个体材料进行了核糖体内转录间隔区 ITS4-5 和叶绿体 *atp*B-*rbc*L 测序。假设杂交个体的 ITS 直接测序结果具明显的叠加性, 之后的克隆测序也分离出两种与假设亲本种相同的序列类型, 因此, 这些假设杂交个体得到了证实。据母系遗传的叶绿体 *atp*B-*rbc*L 测序结果, 穗序橐吾同是它们的质体贡献者, 即虽然这些杂交个体具不同的形态特征, 但都是以棉毛橐吾和穗序橐吾为亲本且以穗序橐吾为质体贡献者的杂交后代。

关键词: 棉毛橐吾; 穗序橐吾; 杂交后代; 核 ITS4-5; 叶绿体 *atp*B-*rbc*L 片段
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Natural Hybrids between *Ligularia vellerea* and *L. subspicata* (Asteraceae: Senecioneae)

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Abstract: Three types of intermediate morphological individuals were found in the sympatric distribution areas of *Ligularia vellerea* and *L. subspicata*. These intermediate individuals were hypothesized to be the hybrids of these two species based on the detailed comparison of their morphological characters. To test this hypothesis, we compared the DNA sequence of *L. vellerea*, *L. subspicata* and the intermediate individuals by using the internal transcribed spacer region of nuclear ribosomal and *atp*B-*rbc*L intergenic spacer region of the chloroplast. Direct sequencing of ITS clearly had additives and then cloned sequencing cloned out two kinds of sequences that were identical to those putative parental species, thus these putative hybrids were approved. With different morphological characters among three types, their plastid donors were all *L. subspicata* according to the maternal sequences of *atp*B-*rbc*L.

Key words: *Ligularia vellerea*; *L. subspicata*; Hybrid progeny; ITS; *atp*B-*rbc*L

There are increasingly voices to illustrate the important role of natural hybridization in plant evolution (Arnold, 1997; Rieseberg, 1997; Rieseberg *et al.*, 2000; Abbott *et al.*, 2008, 2010; Soltis and Soltis, 2009; Chase *et al.*, 2010). The consequences of hybridization may include introgression affecting one or both taxa, formation of hybrid (especially alloploid) species, and development of reticulate pat-

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terns of evolution within a group (Arnold, 1997; Soltis and Soltis, 2009).

The genus *Ligularia* Cass. consists of about 140 species mainly distributed in eastern Asia, one-hundred twenty-four species of which are known in China (Liu, 1989; Liu *et al.*, 1994; Liu and Illarionova, 2011). The majority of *Ligularia* distributed in the Hengduan Mountains area are endemic (Liu *et al.*, 1994; Liu *et al.*, 2006). Interspecific diploid hybridization was considered to be an important cause for the high species diversity in this genus (Liu *et al.*, 2001, 2006). Natural hybrids were commonly found in certain area which *Ligularia* sympatric distributed (Liu *et al.*, 2006; Yu *et al.*, 2011). *Ligularia*×*maoniushanensis* X. Gong & Y. Z. Pan is the first reported natural hybrid species through comprehensive studying (Pan *et al.*, 2008). Furthermore, by comparing *L. lamarum* and *L. subspicata* chemical similarities, Saito *et al.* (2011) presumed that the two species hybridized with other species of this genus commonly. Yu *et al.* (2011) studied one mix population with 6 *Ligularia* species, based on morphological and molecular data (cpDNA and ITS sequences, ISSR markers), bidirectional but asymmetric hybridization and also backcross was detected between *L. subspicata* and *L. nelumbifonia*. Hybridization is very common in *Ligularia* with various form and progenies.

During field collection trips to the Hengduan Mountains in Yunnan, we found a mixed population including *L. subspicata* (Bur. et Franch.) Hand.-Mazz., *L. vellerea* (Franch.) Hand-Mazz., and a putative natural hybrid occurring on Mt. Daxue. The putative nothospecies was considered to be a natural hybrid between *L. subspicata* and *L. vellerea* based on its intermediate morphological appearance and the partial overlap of the flowering period. Given this anthesis overlap, cross-pollination between two putative parents could occur naturally under sympatric conditions.

In the current study, the ITS region of the nuclear ribosomal DNA of all the sampled individuals was sequenced to determine the hybrid status of the morphologically taxon. Once its hybrid status was confirmed, we sequenced chloroplast *atp*B- *rbc*L region for three taxa to determine the relationship between these hybrids and the two natural species.

1 Materials and methods

1.1 Material collection and molecular methods

We collected *Ligularia vellerea*, *L. subspicata* and the putative hybrids in the mix population on the Daxue Mountain, Shangri County, Yunnan, China. Young leaves of 25 individuals (including 10 *L. vellerea*, 10 *L. subspicata* and 5 putative hybrids) were collected and dried in silica gel in field for DNA extraction. Though no other *Ligularia* species was found in this population, a set of other species (sequences download from NCBI, **Appendix**) were included to verify if this population genetic material contaminate by other species in the phylogenetic analysis. Total genomic DNA was extracted from the silica gel-dried leaf tissue using the cetyltrimethyl ammonium bromide (Cruz-Mazo *et al.*, 2009) methods (Doyle and Doyle, 1987) with minor modification.

The ITS region of the nuclear ribosomal DNA of all the sampled individuals was amplified using primers ITS4 and ITS5 (White *et al.*, 1990). PCR was conducted in a total reaction volume of 20 μL, containing 10－30 ng template DNA, 2.0 μL 10× PCR Buffer with $(NH_4)_2SO_4$, 1.3 μL $MgCl_2$ (25 mmol·L^{-1}), 0.5 μL dNTP (2.5 mmol·L^{-1} each), 0.3 mmol·L^{-1} each primer and 0.75 unit of *Taq* polymerase (Takara). Amplifications were performed as following conditions: 1 cycle, 94 ℃, 5 min; 30 cycles, 94 ℃, 45 sec; 55 ℃, 1 min; 72 ℃, 40 sec, followed a final extension of 1 cycle, 72 ℃, 5 min. The PCR products were purified by electro-phoresis through a 1.2% agarose gel followed by use of an E. Z. N. A. Gel Extraction Kit (Omega, Guangzhou, China). All accessions were subjected to sequencing, with amplification primers in an ABI 3700 DNA automated sequencer with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, U. S. A.). The sequences were

aligned and compared in SeqMan (DNASTAR, Beijing, China) (Burland, 1999). While direct sequencing is feasible for *L. vellerea* and *L. subspicata*, it produced chimeric or unreadable peaks in the chromatogram for the putative hybrids. Hence, cloning sequencing was performed subsequently for the putative hybrid. Purified PCR products were cloned into plasmids using the pUM-T vector system (Bioteke). Four to twenty positive clones were selected for each amplification product and cultured for isolating plasmids. Positive clones with the inserts of correct size were confirmed by colony PCR. The plasmids with correct inserts were sequenced using ITS4 and ITS5 primers.

All samples from *L. vellerea*, *L. subspicata* and the putative hybrid were used in the chloroplast *atp*B- *rbc*L. Chloroplast *atp*B- *rbc*L region were amplified using primers *atp*B and *rbc*L (Chiang *et al.*, 1998). PCR was conducted in a reaction volume of 20 μL, containing 10−30 ng template DNA, 2.0 μL 10×PCR Buffer with $(NH_4)_2SO_4$, 1.0 μL $MgCl_2$ (25 mmol · L^{-1}), 1.0 μL dNTP (2.5 mmol · L^{-1} each), 0.3 mmol · L^{-1} each primer and 1.5 unit of *Taq* polymerase (Takara). Amplification conditions were as follows: 1 cycle, 94 ℃, 4 min; 30 cycles, 94 ℃, 45 sec; 53 ℃, 45 sec; 72 ℃, 1 min or 50 sec, followed by 1 cycle, 72 ℃, 7 min. PCR products were purified and then directly sequenced bidirectional using the methods mentioned above.

1.2 Phylogenetic analysis

Minor variation (usually one or two nucleotide sites) between some clones may have resulted from PCR error caused by Taq DNA polymerase. Putative PCR-mediated recombinants were excluded before further phylogenetic analysis. All sequences were aligned in Clustal X (Thompson *et al.*, 1997) complimented PAUP4.0. Information about variable sites was obtained using the program DnaSP 4.0 (Rozas *et al.*, 2003). The aligned sequences were used to infer the phylogeny of the sampled individuals using the criterion of maximum parsimony, implemented in PAUP* version 4.0b (Swofford, 2002). Parsimony analyses were carried out using a heuristic search with tree bisection and reconnection branch swapping, the Multrees option, ACCTRAN optimisation, and 1 000 random addition replicates for the nuclear and chloroplast datasets.

2 Results

2.1 Morphology

Ligularia vellerea and *L. subspicata* belong to Ser. *Scapicaulis* S. W. Liu and Ser. *Ligularia* Cass., respectively (Liu, 1989). The main morphological differences between the two species are leaf shapes, hairs on stems and capitula types (Table 1, Fig. 1). The leaves of *L. vellerea* are ovate with cuneate base, whereas those of *L. subspicata* are cordate or hastate. The stem of *L. vellerea* is full of densely long white hairs, whereas that of *L. subspicata* is proximally glabrous. There are 5−7 ray florets in one capitulum of *L. vellerea*, but none in *L. subspicata*. The putative natural hybrids are intermediate between those of the putative parental species, with ovate or cordate leaves, with/without ray florets in capitula and with/without hairs on stem and the morphological characters show three types of apparent transition state. Type-**a** and type-**b** are similar to *L. vellerea*, but there are thinner hairs on stem in type-a and type-b, and no ray florets in type-b. Type-**c** is similar to *L. subspicata* but with ray florets. The shape form of their leaves show apparently transition state.

2.2 Phylogenetic analysis based on nrDNA ITS4-5

The direct sequence aligned matrix generated a total of 642 bp or 641 bp (because of one indel). No variation was detected within species of *L. vellerea* and *L. subspicata*. There were 15 variation sites between the two putative parental species. The putative natural hybrids had more than ten double peaks, and cloned sequences obtained two sequence types that were identical to those of *L. vellerea* and *L. subspicata*, respectively, which then clearly displaying that all double copies of ITS came from the putative parental species (Table 2).

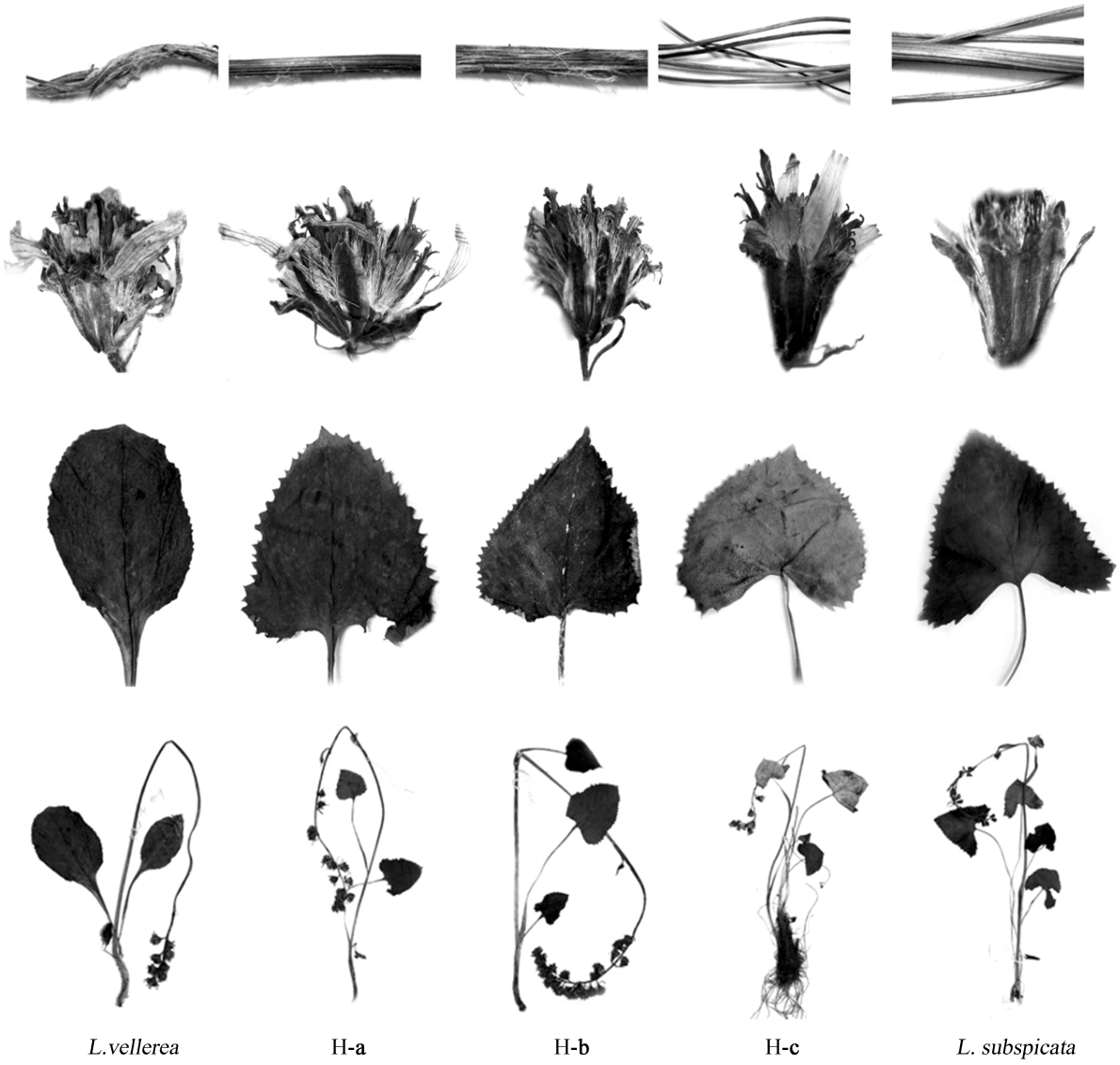


Fig. 1 From up to bottom show stems hairs, florets, laminas and whole individual of putative hybrids and their putative parents respectively. *L. vellerea* show the ovate with cuneate base leaves, 5-7 ray florets in one capitulum and full of densely long white hairs on stems; H-**a** show the cordate leaf, with stem leaves, several ray florets in one capitulum and thinner white hairs on stems; H-**b** show the cordate leaf, with stem leaves, no ray florets in capitulum and densely long white hairs on stems; H-**c** show the cordate leaf, with stem leaves, several ray florets in one capitulum and glabre stems; and *L. subspicata* show the hastate or cordate leaves with stem leaves, no ray florets in one capitulum and glabre stems. Photographs not to scale

Table 1 Main morphological differences characterizing of *Ligularia vellerea*, *L. subspicata* and three types of putative hybrids

Taxa	Characters		
	stems clothing	stem leaves	sexuality of florets composite raceme
Ligularia vellerea	densely long white puberulent	without stem leaf	numerous tubular florets, with several ray florets
putative hybrid **a** (H-**a**)	sparsely long white puberulent	with stem leaves	numerous tubular florets, with several ray florets
putative hybrid **b** (H-**b**)	long white puberulence	with stem leaves	numerous tubular florets, without ray floret
putative hybrid **c** (H-**c**)	proximally glabrous	with stem leaves	numerous tubular florets, with several ray florets
L. subspicata	proximally glabrous	with stem leaves	numerous tubular florets, without ray floret

Table 2　Sites of variation and indels of ITS4-5 from related taxa

Taxa	Polymorphic sites														
	45	10	15	157	18	24	254	27	31	46	48	48	55	56	65
		00	02	-159	00	09	-255	44	99	44	00	55	74	64	66
A1-5	A	T	G	TCC	C	C	GT	G	C	C	C	G	C	C	C
A2-5	A	C	G	TCC	C	C	GT	G	C	T	T	G	C	C	C
A3-1	A	C	G	TCC	C	C	GT	A	T	T	C	G	C	C	C
B1-3	A	C	G	TCC	C	C	GT	G	C	T	T	G	C	C	C
C5-2	A	C	G	TCC	C	C	GT	G	T	C	C	G	C	C	C
S1-S10, S12	A	C	G	TCC	C	C	GT	G	C	T	Y	G	C	C	C
A1	W	Y	R	YYM	Y	-	RY	A	C	C	C	R	Y	Y	C
A2	W	C	R	YYM	Y	C	RY	G	C	Y	Y	R	Y	Y	C
A3	W	C	R	YYM	Y	-	RY	A	C	C	C	R	Y	Y	Y
B1	W	C	R	YYM	Y	C	RY	A	C	Y	Y	R	Y	Y	Y
C5	W	C	R	YYM	Y	-	RY	A	Y	C	C	R	Y	Y	C
V1-V4, V6-V10, V12	T	C	A	CTA	T	-	AC	A	C	C	C	A	T	T	T
A1-1	T	C	A	CTA	T	-	AC	A	C	C	C	A	T	T	T
A2-3	T	C	A	CTA	T	-	AC	A	C	C	C	A	T	T	T
A3-2	T	C	A	CTA	T	-	AC	A	C	C	C	A	T	T	T
B1-6	T	C	A	CTA	T	-	AC	A	C	C	C	A	T	T	T
C5-3	T	C	A	CTA	T	-	AC	A	C	C	C	A	T	T	T

Note: C+T=Y　A+C=M　A+T=W　A+G=R　G+T=K　C+G=S

Taxa: A, H-**a**; B, H-**b**; C, H-**c**; S, *L. subspicata*; D, *L. vellerea*. Numbers following taxon initials are sample numbers and clone numbers (if any).

The strict consensus tree from parsimony analysis of 25 individuals was generated to show phylogenetic relationship among them (Fig. 2). The MP tree indicates that putative parents clustered restively branches with high bootstrap value. With cloned sequences, each individual possessed one copy of one putative parent and clustered to parent branches exactly.

2.3　Phylogenetic analysis based on sequences of cpDNA *atp*B-*rbc*L

Direct sequencing of *atp*B-*rbc*L generated 740 bp for *L. vellerea* and 739 bp for *L. subspicata*, exhibited 4 variation sites which included one indels (Table 3). Among these sites, there were no polymorphisms within *L. subspicata* individuals but one exception in *L. vellerea*. All of putative hybrids had the same *atp*B-*rbc*L sequences with *L. subspicata*.

The MP tree obtained from the cpDNA *atp*B-*rbc*L regions is shown in Fig. 3. The putative parental species formed two separate clusters. All the putative hybrids clustered together with *L. subspicata*. The other *Ligularia* species were clearly separated from the putative parental species and hybrids. Because chloroplast DNA is maternally inherited in *Ligularia* (Zhang *et al.*, 2003), these putative hybrids had *L. subspicata* as the plastid donor. Hence unidirectional hybridization was detected between *L. subspicata* and *L. vellerea*.

Table 3　Sites of variation and indels of chloroplast sequences from related taxa

Taxa	Polymorphic sites			
	28	409	513	681
S1-S10, S12	G	T	-	C
A1	G	T	-	C
A2	G	T	-	C
A3	G	T	-	C
B1	G	T	-	C
C5	G	T	-	C
V1-V4, V6, V7, V9, V10, V12	A	A	-	A
V8	G	A	A	A

Taxa initials and numbering are as in Table 2

3 Discussion

During our field research on *Ligularia* species more than decade, *L. vellerea* and *L. subspicata* are commonly found coexist, but no intermediate morphological individuals were discovered in these sympatric areas except the one on Mt. Daxue. The putative hybrids grow on an open space in a forest edge that was previously dominated by fir. Some trees were destroyed in road building and maintenance, and now grassland mainly grows the putative parental *Ligularia* species appeared on both sides of the road. This easily disturbed micro-habitat may have promoted hybridization between these two species. Such special circumstances are also more likely to give rise to new species of hybrid origin (Buerkle *et al.*, 2000; Rieseberg *et al.*, 2003; Gompert *et al.*, 2006).

Plant grow on wintry highland requires to evolve certain common adaptive mechanisms. *Ligularia* species often share the same pollinators in the highlands (Liu, 2002; Cao *et al.*, 2008). When different species bloom at same place and time, nectar-collecting insects may transfer one species' pollen to another species' flowers casually. The flowering times of the sympatric *Ligularia* species overlap, according to previous

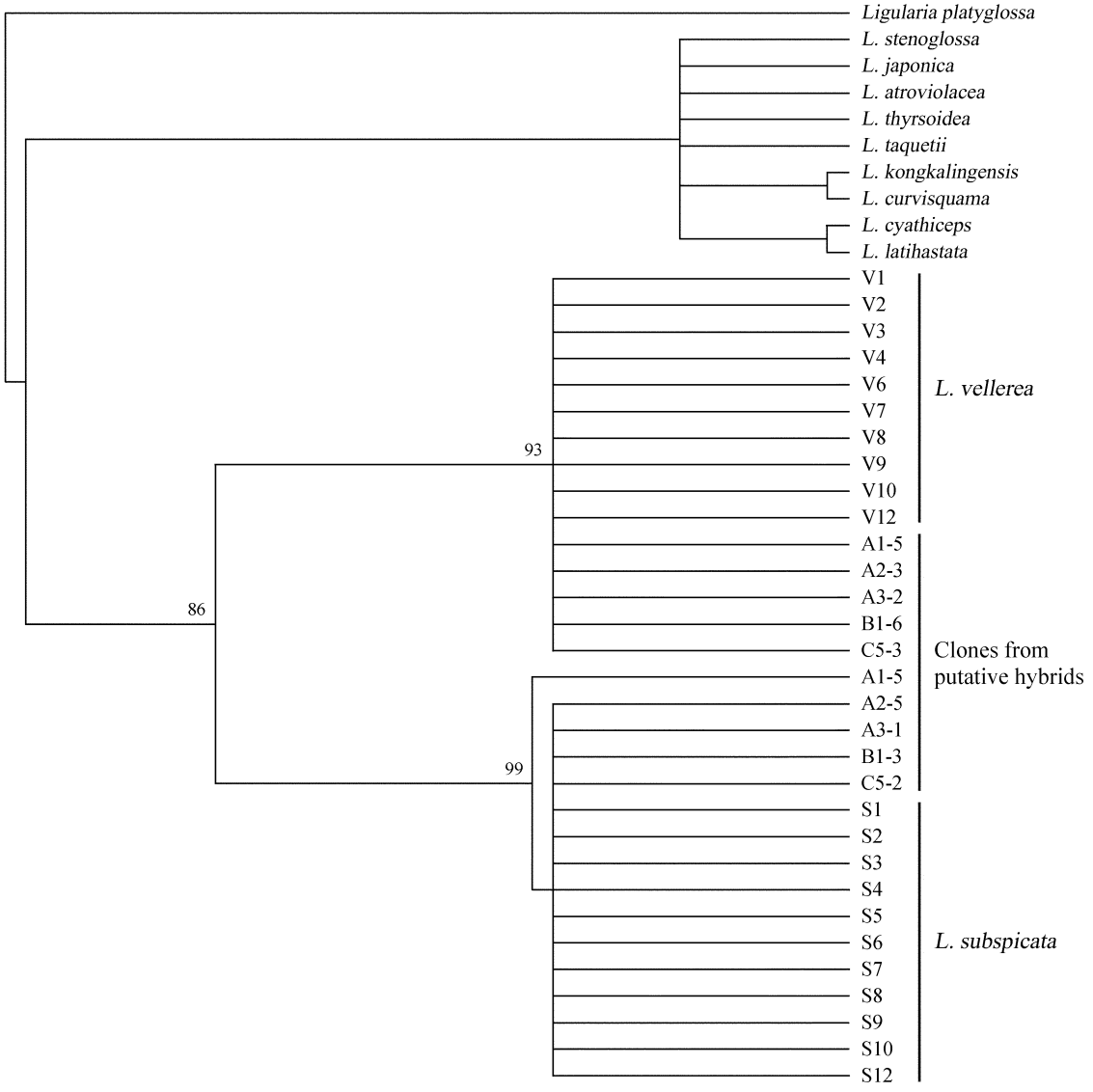


Fig. 2 Strict consensus tree from a parsimony analysis of ITS4-5 sequences from putative hybrid individuals and their putative parental species. Taxa: **V**, *L. vellerea*; **S**, *L. subspicata*; **A**, putative hybrids **a**; **B**, putative hybrids **b**; **C**, putative hybrids **c**. Numbers following taxon initials are sample numbers and clone numbers (if any). Bootstrap percentages >50% are shown above the branches

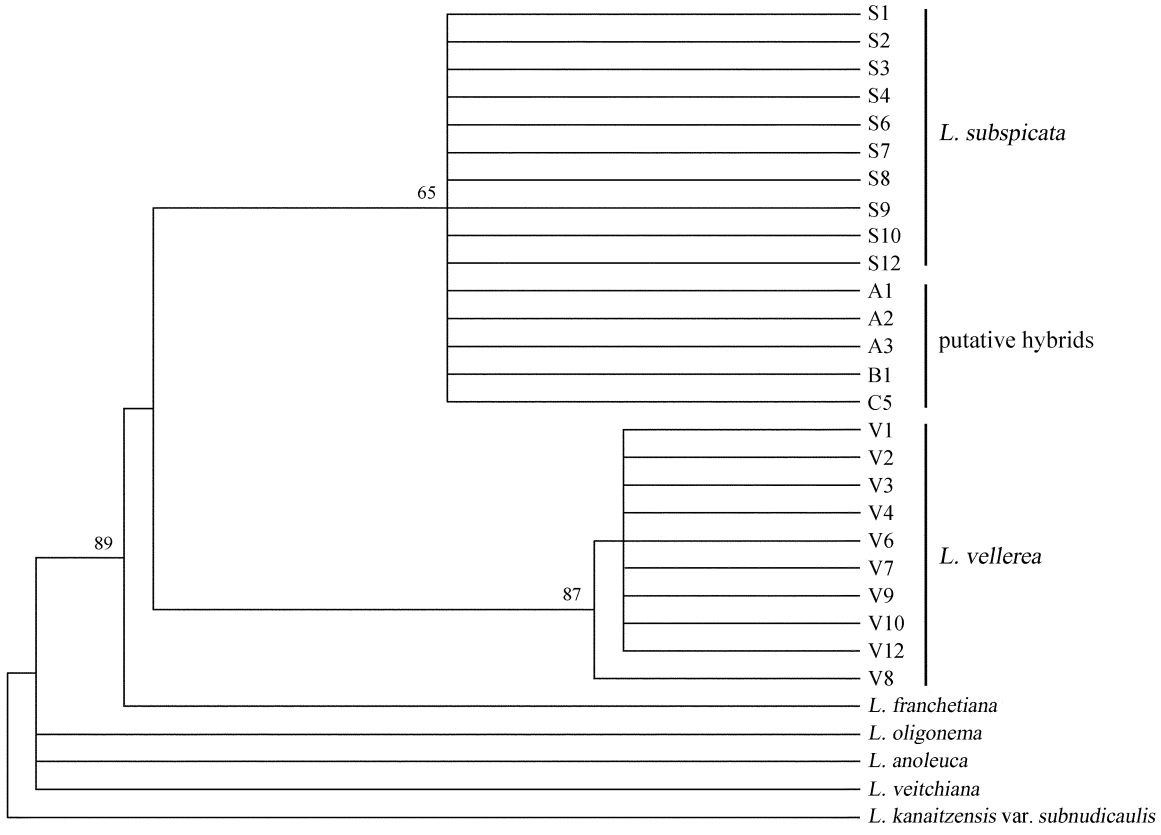


Fig. 3　Maximum parsimony tree inferred from *atp*B-*rbc*L sequences to show the relationships between the putative hybrids and putative parents. Bootstrap percentages >50% are shown above the branches. Taxon initials and numbering are as in Fig. 2

researchers and our field observation for years. They flower in late June to August, but their flowering times differ somewhat: the anthesis *L. vellerea* is a little earlier than *L. subspicata*.

Our ITS4-5 data definitely support the hypothesis that the morphologically intermediate individuals are the progeny of hybridization between *L. vellerea* and *L. subspicata*. According to the results of cpDNA analysis, we can also conclude that the hybridization is unidirectional in the form of *L. subspicata* ♀ × *L. vellerea* ♂.

Three types of putative hybrids were assigned according to their different morphology. We thought this hybridization might bidirectional and parental species might more than the putative two. All of hybrids we studied were more close to parental species of *L. subspicata*. But with some different morphological characters in detail, the first hybrids type H-**a** had *L. vellerea* florets component part with several ray florets except tubular florets in one capitulum, H-**b** had hairs on stem and H-**c** had ray florets. Though they had same plastid donor, they might inherit different copies of nuclear genetic material which leads to different phenotype. Another possibility is F_2, Fns or backcross appeared and hybrid swarm formed at this population. Because materials were limited in the first collection, new and long term observation was needed for further studying.

References:

Abbott RJ, Hegarty MJ, Hiscock SJ *et al.*, 2010. Homoploid hybrid speciation in action [J]. *Taxon*, **59** (5): 1375—1386

Abbott RJ, Ritchie MG, Hollingsworth PM, 2008. Introduction. Speciation in plants and animals: pattern and process [J]. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **363** (1506): 2965—2969

Arnold ML, 1997. *Natural Hybridization and Evolution* [M]. New York: Oxford University Press

Buerkle CA, Morris RJ, Asmussen MA *et al.*, 2000. The likelihood of homoploid hybrid speciation [J]. *Heredity*, **84** (4): 441—451

Burland TG, 1999. DNASTAR's Lasergene sequence analysis software

[J]. *Methods in Molecular Biology*, **132**: 71—91

Cao Y, Ma RJ, Wang GX, 2008. The breeding system of three species of genus *Ligularia* in the east of Qinghai-Tibet Plateau [J]. *Guihaia*, **28** (3): 302—306

Chase MW, Paun O, Fay MF, 2010. Hybridization and speciation in angiosperms: a role for pollinator shifts? [J]. *BMC Biology*, **8** (1): 45

Chiang TY, Schaal BA, Peng CI, 1998. Universal primers for amplification and sequencing a noncoding spacer between the *atp*B and *rbc*L genes of chloroplast DNA [J]. *Botanical Bulletin of Academia Sinica*, **39**: 245—250

Cruz-Mazo G, Buide ML, Samuel R *et al.*, 2009. Molecular phylogeny of *Scorzoneroides* (Asteraceae): Evolution of heterocarpy and annual habit in unpredictable environments [J]. *Molecular Phylogenetics and Evolution*, **53** (3): 835—847

Doyle JJ, Doyle JL, 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue [J]. *Phytochemical Bulletin*, **19** (1): 11—15

Gompert Z, Fordyce JA, Forister ML *et al.*, 2006. Homoploid hybrid speciation in an extreme habitat [J]. *Science*, **314** (5807): 1923—1925

Liu JQ, Liu SW, Ho TN *et al.*, 2001. Karyological studies on the Sino-Himalayan genus, *Cremanthodiurn* (Asteraceae: Senecioneae) [J]. *Botanical Journal of the Linnean Society*, **135** (2): 107—112

Liu JQ, Wang YJ, Wang AL *et al.*, 2006. Radiation and diversification within the *Ligularia-Cremanthodium-Parasenecio* complex (Asteraceae) triggered by uplift of the Qinghai-Tibetan Plateau [J]. *Molecular Phylogenetics and Evolution*, **38** (1): 31—49

Liu SW, 1989. Compositae-Senecioneae [A]. In: Ling Y, Liu SW (eds.), *Flora Reipublicae Popularis Sinicae* [M]. Beijing: Science Press, 4—115

Liu SW, Deng DS, Liu JQ, 1994. Origin, evolution and distribution of *Ligularia* Cass. (Compositae) [J]. *Acta Phytotaxonomica Sinica*, **32**: 514—525

Liu SW, Illarionova ID, 2011. Ligularia [A]. In: Wu ZY, Raven PH, Hong DY (eds.), *Flora of China* [M]. Beijing: Science Press; St. Louis: Missouri Botanical Garden Press, 371—544

Liu ZJ, 2002. Life History Strategies of Ligularia virgaurea, an Advantage Toxic Forb in Degradative Rangland of Alpine Meadow (Doctor degree) [D]. Lanzhou: Lanzhou University China

Pan YZ, Shi SH, Gong X *et al.*, 2008. A natural hybrid between *Ligularia paradoxa* and *L. duciformis* (Asteraceae, Senecioneae) From Yunnan, China [J]. *Annals of the Missouri Botanical Garden*, **95** (3): 487—494

Rieseberg LH, 1997. Hybrid origins of plant species [J]. *Annual Review of Ecology and Systematics*, **28** (1): 359—389

Rieseberg LH, Baird SJE, Gardner KA, 2000. Hybridization, introgression, and linkage evolution [J]. *Plant Molecular Biology*, **42** (1): 205—224

Rieseberg LH, Raymond O, Rosenthal DM *et al.*, 2003. Major ecological transitions in wild sunflowers facilitated by hybridization [J]. *Science*, **301** (5637): 1211—1216

Rozas J, Sanchez-DelBarrio JC, Messeguer X *et al.*, 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods [J]. *Bioinformatics*, **19** (18): 2496—2497

Saito Y, Hattori M, Iwamoto Y *et al.*, 2011. Overlapping chemical and genetic diversity in *Ligularia lamarum* and *Ligularia subspicata*. Isolation of ten new eremophilanes and a new seco-bakkane compound [J]. *Tetrahedron*, **67** (12): 2220—2231

Soltis PS, Soltis DE, 2009. The role of hybridization in plant speciation [J]. *Annual Review of Plant Biology*, **60**: 561—588

Swofford DL, 2002. *PAUP**: *Phylogenetic Analysis Using Parsimony, Version 4.0 b10* [M]. Sunderland, MA: Sinauer Associates

Thompson JD, Gibson TJ, Plewniak F *et al.*, 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools [J]. *Nucleic Acids Research*, **25** (24): 4876—4882

White TJ, Bruns T, Lee S *et al.*, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics [J]. *PCR Protocols: A Guide to Methods and Applications*, **18**: 315—322

Yu JJ, Kuroda C, Gong X, 2011. Natural hybridization and introgression in sympatric *Ligularia* species (Asteraceae, Senecioneae) [J]. *Journal of Systematics and Evolution*, **49** (5): 438—448

Zhang Q, Liu Y, Sodmergen, 2003. Examination of the cytoplasmic DNA in male reproductive cells to determine the potential for cytoplasmic inheritance in 295 angiosperm species [J]. *Plant and Cell Physiology*, **44** (9): 941—951

Appendix: List of accessions of *Ligularia* included in the phylogenetic analysis of nuclear rDNA ITS and *atp*B-*rbc*L sequences. GenBank numbers for all accessions are provided.

Accessions, GenBank No.

ITS: *Ligularia platyglossa*, AY458841; *L. stenoglossa*, AB523365; *L. japonica*, AY458825; *L. atroviolacea*, DQ272322; *L. thyrsoidea*, DQ272320; *L. taquetii*, GU323282; *L. kongkalingensis*, DQ272343; *L. curvisquama*, DQ272324; *L. cyathiceps*, DQ272328; *L. latihastata*, DQ272334.

***atp*B-*rbc*L**: *L. franchetiana*, AB375447; *L. oligonema*, AB523847; *L. anoleuca*, AB557885; *L. veitchiana*, AB557887; *Ligularia kanaitzensis* var. *subnudicaulis*, AB281495.